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Biochemistry

Control of pre-mRNA accumulation by the essential yeast protein Nrd1 requires high-affinity transcript binding and a domain implicated in RNA polymerase II association

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ABSTRACT **Nrd1 is an essential yeast protein of unknown function that has an RNA recognition motif (RRM) in its carboxyl half and a putative RNA polymerase II-binding domain, the CTD-binding motif, at its amino terminus. Nrd1 mediates a severe reduction in pre-mRNA production from a reporter gene bearing an exogenous sequence element in its intron. The effect of the inserted element is highly sequence-specific and is accompanied by the appearance of 3′-truncated transcripts. We have proposed that Nrd1 binds to the exogenous sequence element in the nascent pre-mRNA during transcription, aided by the CTD-binding motif, and directs 3′-end formation a short distance downstream. Here we show that highly purified Nrd1 carboxyl half binds tightly to the RNA element *in vitro* with sequence specificity that correlates with the efficiency of cis-element-directed down-regulation *in vivo*. A large deletion in the CTD-binding motif blocks down-regulation but does not affect the essential function of Nrd1. Furthermore, a nonsense mutant allele that produces truncated Nrd1 protein lacking the RRM has a dominant-negative effect on down-regulation but not on cell growth. Viability of this and several other nonsense alleles of Nrd1 appears to require translational readthrough, which in one case is extremely efficient. Thus the CTD-binding motif of Nrd1 is important for pre-mRNA down-regulation but is not required for the essential function of Nrd1. In contrast, the RNA-binding activity of Nrd1 appears to be required both for down-regulation and for its essential function.**

We recently reported the discovery and characterization of an essential yeast gene, *NRD1*, whose protein product has features suggestive of an involvement in the earliest stages of expression of genes transcribed by RNA polymerase II (Pol II) (1). These features include a ribonucleoprotein consensus type RNA recognition motif (RRM) and a segment containing several arginine-glutamate and arginine-serine (RE/RS) dipeptides, similar to those found in many metazoan splicing factors. At its amino terminus, Nrd1 shares sequence identity with mammalian proteins identified on the basis of their interaction with the repetitive carboxyl-terminal domain (CTD) of the largest subunit of RNA Pol II, and this amino-terminal portion of Nrd1 binds the mouse RNA Pol II CTD *in vivo* as judged by two-hybrid analysis (2).

Our studies showed that Nrd1 mediates the severe reduction in expression of a reporter gene resulting from the introduction of an exogenous sequence element into its intron (1). In a wild-type strain, very little full-length reporter pre-mRNA accumulates, and truncated pre-mRNA fragments having endpoints downstream of the intronic sequence element are produced. This effect is suppressed either by point mutations in a short segment of the exogenous cis element or by mutations in *NRD1*, including nonsense mutations upstream of the RRM or a missense mutation in the RRM. We proposed that Nrd1 binds by means of its RRM directly to pre-mRNA containing the sequence element, and that this binding is a prerequisite for truncation of the pre-mRNA. We further hypothesized that interaction of Nrd1 with the CTD of RNA Pol II might facilitate the cotranscriptional recognition of this element, such that recognition occurs before the intron is excised from the pre-mRNA by splicing. The viability of several *nrd1* alleles containing early nonsense codons suggested that the amino-terminal domain of Nrd1 provides its essential function, possibly by modulating the elongation activity of RNA Pol II (1, 3).

Here we investigate several key features of our model. We provide strong biochemical support for the hypothesis that sequence-specific recognition of the intronic sequence element by the carboxyl-terminal half of Nrd1 is required for down-regulation. We also find that the CTD-binding motif of Nrd1 plays an important role in cis-element-directed pre-mRNA down-regulation, probably by mediating protein–protein interactions. However, although a deletion removing much of the CTD-binding motif results in loss of cis-element-directed down-regulation, the resulting allele is fully viable. Viability of cells carrying the *nrd1-3* allele, which has a premature translation termination signal immediately after the CTD-binding motif, is dependent on the remainder of the Nrd1 reading frame. We present evidence that the *nrd1-3* stop codon is read through with very high efficiency, producing nearly normal levels of full-length protein. Together, these results indicate that the amino-terminal domain of Nrd1 implicated in interactions with the RNA Pol II CTD is required for reporter pre-mRNA down-regulation but dispensable for viability, and that the essential function of Nrd1 is mediated by the carboxyl-terminal part of the protein having RNA-binding activity.

MATERIALS AND METHODS

Yeast Strains and Methods. Yeast methods and strains 46α and EJS101 were described previously (1). EJS101–9d is a haploid progeny of EJS101 that carries the *HIS3*-marked disruption of the chromosomal *NRD1* locus and the *URA3*-marked centromeric plasmid, pRS316NRD1. Fragments containing the *NRD1* gene or mutant alleles were cloned into pRS314 (CEN plasmid, *TRP1*) and pRS424 (2μ plasmid, *TRP1*). pRS314NRD1Δ39–169 was constructed by digestion with *Bcl*I and *Bam*HI followed by ligation of the compatible

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Abbreviations: CTD, carboxyl-terminal domain of the largest subunit of RNA polymerase II; RRM, RNA recognition motif; Pol II, polymerase II; ddATP, 2′,3′-dideoxy-ATP; ddCTP, 2′,3′-dideoxy-CTP.

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termini. pRS314NRD1-3ΔBam-Bgl was constructed by digestion with *Bam*HI and *Bgl*II followed by ligation, which removes the entire Nrd1 coding region downstream of codon 169 while leaving the 3′ untranslated region intact. These plasmids were introduced into EJS101–9d for viability tests on plates containing 5-fluoroorotic acid (5-FOA) to select against pRS316NRD1. Plasmids were also introduced into the *cup1*Δ strain 46α carrying the ACT-CUP reporter plasmid pGAC24-U6R*ΔNru and were tested for dominant suppression of ACT-CUP mRNA down-regulation on plates containing copper.

Protein Expression and Purification. A *Bam*HI site overlapping codon 307 of Nrd1, introduced by PCR, was used to subclone a *Bam*HI–*Eco*RI fragment encoding residues 307–560 of Nrd1 into *Bam*HI–*Eco*RI-digested pET21b (Novagen), generating pET21b-Nrd1$_{307–560}$. This plasmid encodes a fusion protein with an amino-terminal T7 leader peptide and 17 carboxyl-terminal residues, including six tandem histidine residues at the extreme carboxyl terminus, all derived from the vector (Fig. 1).

Expression of recombinant protein in *Escherichia coli* strain BL21(DE3) was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 3 hr at 37°C. Cell pellet from a 200-ml induced culture was resuspended (total volume = 4 ml) in buffer A (500 mM NaCl/20 mM Tris·HCl, pH 8.0/5 mM 2-mercaptoethanol) containing 30 mM imidazole and 10 mg/ml lysozyme. After several freeze–thaw cycles, extract was prepared by sonication and cleared by microcentrifugation for 15 min. Extract (≈3 ml) was stirred with 1 ml Ni-NTA resin (Qiagen; NTA is nitrilotriacetate) at 4°C for 1 hr in buffer A containing 30 mM imidazole. The resin was loaded onto a disposable 2-ml column and washed with 10 ml of buffer A/30 mM imidazole, then 4 ml of buffer A/10% (vol/vol) glycerol/30 mM imidazole. Protein was eluted with 6 ml of buffer A/10% glycerol/80 mM imidazole. Peak fractions were concentrated in Centricon-10 concentrators (Amicon) before application to a Sephadex G-100 gel-filtration column equilibrated in buffer B (100 mM NaCl/20 mM Tris·HCl, pH 8.0/10% glycerol/0.1 mM DTT/0.1 mM EDTA). Fractions judged to be free of contaminating proteins (based on Coomassie blue staining after SDS/PAGE) were pooled, concentrated, and stored in buffer B. The concentration of protein was determined spectrophotometrically using a molar extinction coefficient at 280 nm of 35,800, calculated from its inferred amino acid composition (4).

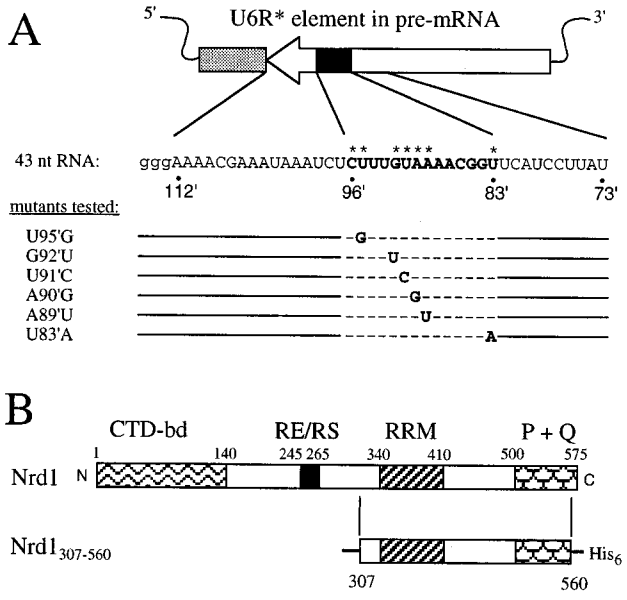

FIG. 1. RNAs and recombinant Nrd1 protein used for RNA-binding assays. (*A*) Schematic diagram of U6R* element that confers Nrd1-dependent RNA down-regulation (1). The arrow represents antisense U6, the shaded box represents the 5′-flanking element, and the black box a 14-nt region of antisense U6 defined by point-mutant suppressors of down-regulation. The sequence of the 43-nt wild-type transcript is shown with three guanosine residues contributed by the T7 promoter in lowercase, and the location and identity of the point mutants analyzed here are shown below. (*B*) Schematic diagram of wild-type Nrd1 and recombinant Nrd1$_{307–560}$ protein. Domains indicated are: CTD-bd, domain implicated in binding to RNA Pol II CTD; RE-RS, arginine-glutamate and arginine-serine dipeptide-rich region; RRM, RNA recognition motif; P+Q, proline- and glutamine-rich region. Sequences contributed to Nrd1$_{307–560}$ by the pET21b expression vector are indicated by the lines at the amino (N) and carboxyl (C) termini.

***In Vitro* RNA Synthesis.** Templates for transcription by T7 RNA polymerase were generated by amplification of portions of the U6R* element from pGAC24-U6R* and cis-suppressor mutants (1). For the 43-nt RNAs, primers used were T7-U6R (5′-TAATACGACTCACTATAGGGAAAACGAAATAA-ATCTC-3′) and U6–5′Xho (1). For the 68-nt RNAs containing the 5′-flanking sequence, T7-U6R* (5′-TAATACGACT-CACTATAGGGATCCACATGTAGTTGCGAGATCCG-3′) was used with U6–5′Xho. Products were digested with *Eco*NI and transcribed with T7 RNA polymerase in the presence 400 μM ATP, CTP, and GTP, 100 μM UTP, and 10 μCi of [α-^{32}P]UTP (1 μCi = 37 kBq). Gel-purified RNAs were quantitated by scintillation counting, using the calculated specific activity of the RNA based on its U content and the experimentally determined specific activity of UTP in the transcription reaction. "Cold" RNAs for competition experiments were trace-labeled with low-specific-activity [α-^{32}P]UTP to allow purification and quantitation.

RNA-Binding Assays. RNA binding reactions were performed in 12 μl of buffer B containing 4 mM $MgCl_2$, ^{32}P-labeled RNA at 1 nM, 40 μg/ml BSA, and indicated concentrations of Nrd1$_{307–560}$ protein. Two microliters of protein at 6 times its final assay concentration in buffer B was mixed with 10 μl of RNA in buffer B/4.8 mM $MgCl_2$ and was incubated at room temperature for 20 min. Competition experiments were carried out in the same fashion, but contained ^{32}P-labeled RNA at 3 nM, protein at 10 nM, and trace-labeled competitor RNA as indicated. Ten-microliter samples were loaded directly onto 5% polyacrylamide gels [19:1 acrylamide/bisacrylamide; 50 mM Tris/50 mM borate/1 mM EDTA, pH 8.3); 15 cm × 15 cm × 1.5 mm] that had been prerun for 1 hr at 200 V in a 4°C cold room, and run for 1 hour at 200 V. Gels were dried onto Whatman 3MM paper and exposed to a PhosphorImager screen, and results were analyzed on a Molecular Dynamics PhosphorImager by using IMAGEQUANT software. Experiments with mutant RNAs were performed in parallel with wild-type RNA on the same gel.

Preparation and Analysis of Yeast RNA. Total RNA was prepared from yeast cells by using the guanidine isothiocyanate/glass bead method (5). Primer extension analysis of the ACT-CUP mRNA was performed with ^{32}P-labeled 3′-CUP, U5B, and U6D oligonucleotides as described previously (1). For analysis of *nrd1-3* mRNA, the primer was Nrd1-Bam169 (5′-GCTTGGGATCCAGTGATA-3′), reactions were scaled up 4-fold, and samples were electrophoresed on 15% polyacrylamide gels. 2′,3′-Dideoxy-ATP (ddATP) and 2′,3′-dideoxy-CTP (ddCTP) were substituted for dATP and dCTP as indicated.

Anti-Nrd1 Antiserum and Immunological Methods. Rabbit antiserum against Nrd1 protein was raised in the Animal Care Unit of the University of Wisconsin Medical School. Initial immunization was with recombinant Nrd1 protein prepared as described for Nrd1$_{307–560}$, except that it included residues 169–560 of the Nrd1 protein. Subsequent boosts were done with Nrd1$_{307–560}$ protein.

For immunoblots, 0.5 OD_{600} unit of yeast cultures growing logarithmically at 23°C in YEPD (yeast extract/peptone/dextrose) were pelleted by microcentrifugation, and cells were washed in H_2O and then boiled in SDS/PAGE loading buffer for 5 min. Samples were electrophoresed on SDS/10% polyacrylamide gels and electroblotted onto Optitran nitrocellulose membranes (Schleicher & Schuell). Blots were blocked with 3% BSA in Tris-buffered saline (TBS; 150 mM NaCl/100 mM Tris·HCl, pH 7.5) with 0.1% Tween-20 for 1 hr, and incubated with Nrd1 antiserum at 1:1000 dilution in TBS/BSA/Tween for 1 hr at room temperature. After being washed three times for 20 min in TBS/Tween, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Tropix; Bedford, MA) at 1:5000 dilution in TBS/Tween for 1 hr, then washed three times in TBS/Tween and once in TBS. Blots were developed by using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma) as chromogenic substrates for alkaline phosphatase.

RESULTS

The Carboxyl-Terminal Half of Nrd1 Binds Sequence-Specifically to the U6R* RNA Element. An artificial sequence element that confers Nrd1-dependent pre-mRNA down-regulation is composed of antisense U6 RNA combined with an unrelated 5′ flanking element, and is called U6R* (Fig. 1*A*). Although the entire element is required for down-regulation, clustered point-mutant suppressors defined a critical 14-nt segment of the antisense U6 portion of the element (1). Because several alleles of *nrd1* that suppress down-regulation are predicted to disrupt function of an RRM, we used purified recombinant Nrd1 protein to test the hypothesis that Nrd1 binds directly to RNA containing this 14-nt segment.

Initial attempts to express full-length Nrd1 protein in *E. coli* were unsuccessful. Therefore, we subcloned a portion of the *NRD1* gene encoding amino acid residues 307–560 into the *E. coli* expression vector pET21b to produce a portion of Nrd1 that includes the RRM for *in vitro* analysis of RNA binding activity. The resulting polypeptide, called $Nrd1_{307–560}$, also contains most of the proline- and glutamine-rich carboxyl-terminal domain as well as a six-histidine tag to facilitate purification (see Fig. 1*B*). Recombinant $Nrd1_{307–560}$ protein was purified to apparent homogeneity by Ni-NTA affinity chromatography and gel filtration.

Using an electrophoretic gel-mobility-shift assay, we examined the binding of $Nrd1_{307–560}$ to *in vitro* synthesized RNAs derived from the U6R* element. A retarded complex of discrete mobility was resolved when a 43-nt RNA containing the 14-nt segment defined by suppressor point mutations was incubated with $Nrd1_{307–560}$, indicating that this RNA contains a single high-affinity binding site for Nrd1 (Fig. 2*A*). We estimate a dissociation constant (K_d) of approximately 10 nM for this interaction (see Fig. 3*B*).

We tested the sequence specificity of Nrd1 binding by using suppressor point mutant RNAs. Mutations located near the center of the 14-nt suppressor mutant cluster have the strongest effect, with the A90′G, U91′C, and G92′U mutant RNAs failing to be assembled into specific complexes even at the highest protein concentrations tested (320 nM; Fig. 2*A* and data not shown). A nonspecific complex of lower mobility is seen at the highest protein concentrations with all RNAs. The other point mutant RNAs tested (U83′A, A89′U, U95′G) each yielded a discrete complex with mobility indistinguishable from that formed with the wild-type RNA (Fig. 2*A*). Binding to the A89′U and U95′G mutants was substantially weaker than to the wild-type sequence, with apparent K_d values of >100 nM and $\approx$40–50 nM, respectively (see Fig. 3*B*). Binding

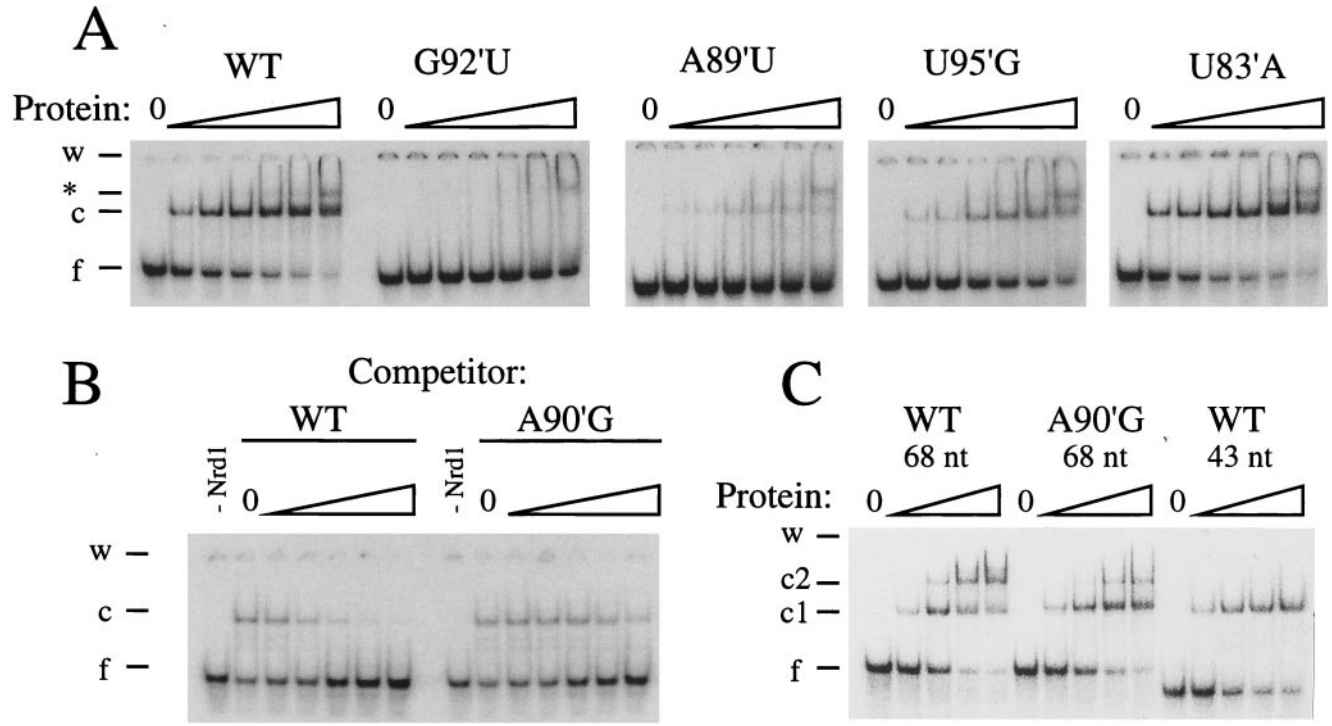

FIG. 2. Gel-mobility-shift assays for RNA binding by $Nrd1_{307–560}$. (*A*) Representative gels showing retardation of wild-type, G92′U, A89′U, U95′G, and U83′A RNAs by binding to $Nrd1_{307–560}$. Protein concentrations assayed were 0, 5, 10, 20, 40, 80, and 160 nM, increasing from left to right for each RNA. Positions of the well, RNA–protein complex, and free RNA are identified by marks labeled w, c, and f, respectively, and the asterisk marks a nonspecific complex observed at the highest protein concentrations. (B) Competition assay for RNA binding to Nrd1, using unlabeled wild-type or A90′G mutant RNA as competitors for binding to labeled 43-nt wild-type RNA. Competitor RNA concentrations were 0, 3, 15, 75, 150, and 300 nM, increasing from left to right. The leftmost lane in each set (− Nrd1) contained no Nrd1 protein. (C) Formation of two discrete complexes on 68-nt RNAs containing the 5′-flanking portion of the U6R* element. Protein concentrations were 0, 10, 20, 40, and 80 nM. The mobilities of complexes interpreted to have 1 or 2 sites on the RNA occupied by Nrd1 are indicated by c1 and c2, respectively.

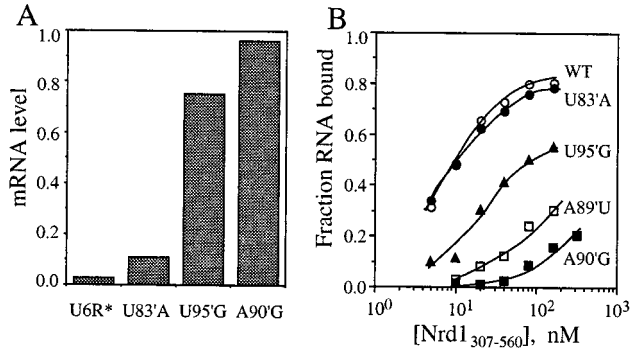

FIG. 3. Correlation between the affinity of $Nrd1_{307–560}$ for 43-nt RNAs *in vitro* and down-regulation of ACT-CUP mRNA *in vivo*. (*A*) Steady-state levels of mRNA produced from ACT-CUP fusion genes with the U6R* insert or selected point mutant inserts. The mRNA level from the wild-type ACT-CUP fusion gene (no U6R* insert) is defined as 1.0. RNA levels are averages from two primer extension experiments, normalized to internal controls provided by extension of U5 and U6 RNAs. (*B*) Binding curves for 43-nt wild-type and selected mutant RNAs. Data are from representative single experiments.

of the U83′A mutant was nearly indistinguishable from that of the wild-type RNA.

Competition experiments with unlabeled wild-type and A90′G mutant RNAs confirmed that the gel-mobility-shift assay accurately reflects the relative affinity of Nrd1 for different RNAs in solution. Comparable levels of competition for binding to labeled RNA were seen, for example, in the presence of 15 nM wild-type competitor or 150 nM A90′G mutant competitor RNAs (Fig. 2*B*).

Deletion analysis demonstrated that in addition to the region of the U6R* element defined by suppressor point mutations, a 5′ element flanking the antisense U6 sequence plays an important role in Nrd1-dependent pre-mRNA control (1). Therefore, we examined the binding of $Nrd1_{307–560}$ to a 68-nt RNA, extended to include this 5′ flanking element. At low protein concentrations, this longer RNA was incorporated into a single discrete complex having a mobility similar to that of the complex formed on the 43-nt RNA (Fig. 2*C*). With increasing protein concentration, a second complex of lower mobility was resolved, suggesting that this RNA contains at least two discrete binding sites for Nrd1 (see *Discussion*). In the context of this 68-nt RNA, formation of the first complex was not altered by the A90′G mutation. Formation of the second, lower-mobility, complex, however, was substantially reduced. Thus the additional binding site has an affinity for Nrd1 similar to that of the site containing A90′.

We quantitated ACT-CUP reporter gene mRNA levels in strains carrying several different cis-suppressor mutant U6R* elements by primer extension. The results, illustrated in Fig. 3*A*, indicate that the efficiency of Nrd1-mediated down-regulation correlates well with the affinity of Nrd1 for sequence variants of the 43-nt RNA *in vitro* (Fig. 3*B*). We conclude that the mechanism of cis suppression is interference with Nrd1 binding to the cis element.

An Amino-Terminal Domain of Nrd1 Implicated in Binding to RNA Pol II Is Dispensable for Viability but Required for Pre-mRNA Down-Regulation. Because disruption of *NRD1* is lethal, the viability of several *nrd1* mutant alleles that contain early nonsense codons suggested that the amino terminus of Nrd1 is sufficient for the essential function (1). Among these alleles, the *nrd1-3* mutant encodes the smallest predicted product at 163 residues, roughly coinciding with the amino-terminal domain of Nrd1 implicated in direct interactions with the RNA Pol II CTD (Fig. 4*A*). This finding suggested that the interaction of Nrd1 with the CTD mediates a function that is necessary for viability of yeast, and that the RNA-binding

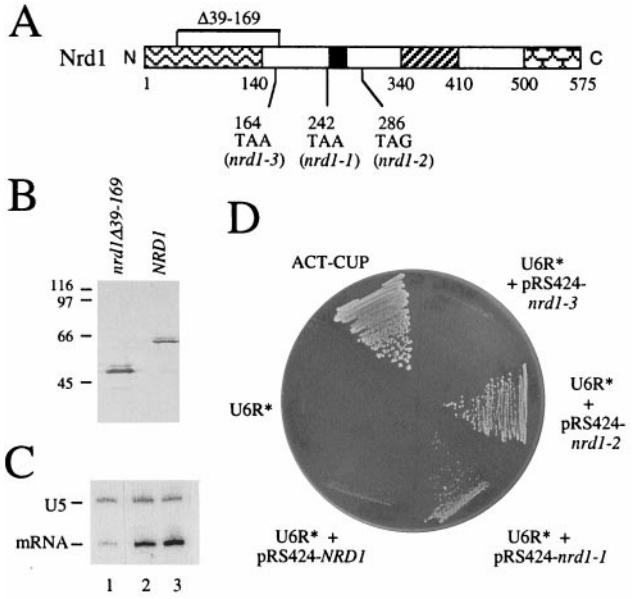

FIG. 4. An amino-terminal domain of Nrd1 is dispensable for viability but required for U6R*-directed RNA down-regulation. (*A*) Schematic of Nrd1 protein (see Fig. 1*B*), showing location of nonsense codons in the *nrd1-1*, *nrd1-2*, and *nrd1-3* alleles. The location of the Δ39–169 deletion is also indicated. (*B*) Immunoblot showing expression of Nrd1Δ39–169 and wild-type Nrd1 proteins from centromere plasmids in the *nrd1Δ39-575* strain, EJS101–9d. Numbers to the left indicate the sizes, in kDa, of molecular mass markers. (*C*) Primer extension analysis of ACT-CUP mRNA, showing suppression of U6R*-directed down-regulation by the *nrd1Δ39-169* allele in EJS101–9d. Products from reverse transcription of ACT-CUP mRNA and U5 RNA are indicated. Lane 1, expression of mRNA from the U6R*-containing ACT-CUP fusion gene in the presence of wild-type *NRD1* allele on a CEN plasmid; lane 2, expression of mRNA from the U6R*-containing fusion gene in the presence of *nrd1Δ39-169*; lane 3, expression of mRNA from the wild-type ACT-CUP fusion gene in the presence of wild-type *NRD1*. (*D*) Copper plate assay for ACT-CUP expression, showing dominant suppression of down-regulation by overexpressed *nrd1-1* and *nrd1-2* in the wild-type *NRD1* strain, 46α. The plate shown contained 0.5 mM $CuSO_4$.

function in the carboxyl-terminal half of the protein is dispensable for viability.

To examine the role of the amino-terminal domain in pre-mRNA down-regulation, we deleted a *Bcl*I–*Bam*HI fragment from the *NRD1* gene, precisely removing codons 39–169. This deletion removes the region of greatest similarity to the mammalian CTD-binding proteins rA4 and rA8 (1, 2). Surprisingly, the resulting *nrd1Δ39-169* allele confers viability to a *nrd1Δ39-575* strain when provided on a low-copy (CEN) plasmid. Immunoblot analysis confirmed that a correspondingly smaller form of Nrd1 accumulates in this strain, at a level comparable to that of the wild-type protein (Fig. 4*B*). Expression of the U6R*-containing reporter mRNA was restored to nearly wild-type levels in this deletion mutant (Fig. 4*C*), indicating that the portion of the protein between residues 39 and 169 plays an important role in down-regulation of U6R*-containing pre-mRNA.

If the amino terminus of Nrd1 mediates a saturable physical interaction required for pre-mRNA down-regulation, then competition for this interaction by overexpressed amino-terminal fragments of the protein lacking RNA-binding activity should interfere with RNA down-regulation by full-length Nrd1. Indeed, expression of *nrd1-1* and, especially, *nrd1-2* from high-copy (2μ) plasmids in a *NRD1* strain enables increased expression of the U6R*-containing ACT-CUP reporter gene, as measured by copper-resistant growth (Fig. 4*D*). Failure of the *nrd1-3* allele to confer dominant suppression when over-

expressed is expected, given the efficient readthrough of its premature stop codon (see below). Importantly, while overexpression of *nrd1-1* or *nrd1-2* interferes with the down-regulation function of wild-type Nrd1, no other phenotypic consequences are observed. We conclude that the amino-terminal portion of Nrd1 mediates physical interactions that are required for U6R*-directed pre-mRNA down-regulation, but dispensable for the essential cellular function of Nrd1.

Efficient Translational Readthrough of a Premature Stop Codon in *nrd1-3*. Given that *nrd1-3*, a viable allele, is predicted to encode only the amino-terminal 163 residues of Nrd1, the finding that deletion of codons 39–169 also results in a viable allele presented a conundrum. This puzzle was further compounded by the results presented in Fig. 5*A*: although the chromosomal *nrd1-1*, *nrd1-2,* and *nrd1-3* alleles all support temperature-sensitive viability of the original strain in which they were selected, only *nrd1-3* is able to support robust growth

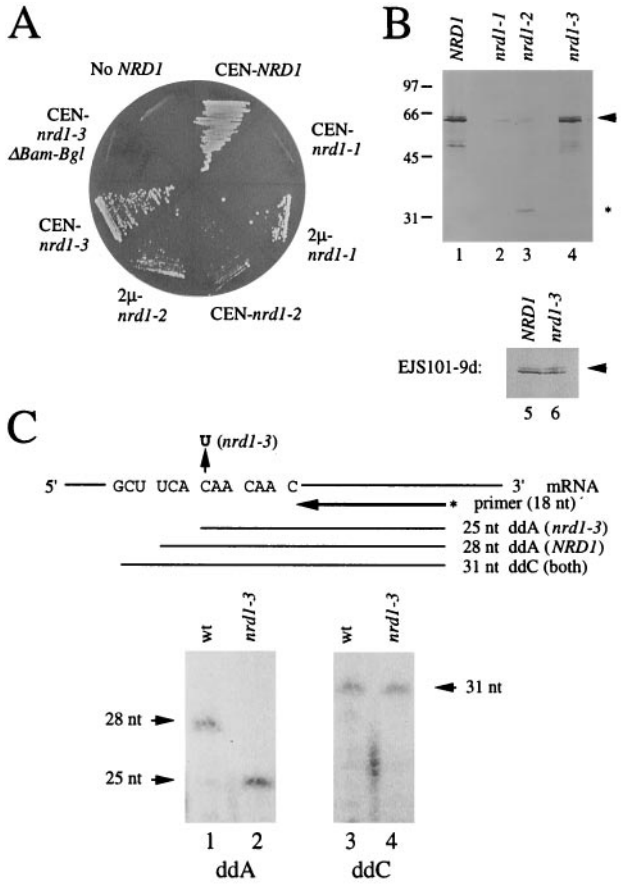

FIG. 5. Expression and function of *nrd1* nonsense alleles. (*A*) Viability of *nrd1* alleles provided on CEN and 2μ plasmids in the *nrd1*Δ strain, EJS101–9d. The plate shown contained 5-fluoroorotic acid to select for loss of the *URA3*-marked plasmid carrying wild-type *NRD1* (pRS316NRD1). (*B*) Immunoblots showing accumulation of Nrd1 protein in strains expressing *nrd1* nonsense alleles. (*Upper*) Lane 1, wild-type *NRD1* strain, 46α; lane 2, *nrd1-1* strain; lane 3, *nrd1-2* strain; and lane 4, *nrd1-3* strain. The arrowhead indicates full-length Nrd1 protein, and the asterisk indicates the truncated product of *nrd1-2*. (*Lower*) Lane 5, *nrd1*Δ strain EJS101–9d expressing wild-type *NRD1* from a CEN plasmid; lane 6, *nrd1*Δ strain expressing *nrd1-3* from a CEN plasmid. (*C*) Primer extension analysis of *nrd1-3* mRNA. The diagram shows the RNA sequence surrounding the *nrd1-3* stop codon and indicates the expected reverse transcription products from wild-type and *nrd1-3* mRNAs. Extension of the Nrd1-Bam169 primer in the presence of ddATP (lanes 1 and 2) shows the presence of a U residue in the mRNA at the position corresponding to the CAA to TAA mutation in the *nrd1-3* gene. Extension in the presence of ddCTP (lanes 3 and 4) yields the same product from both the wild-type and *nrd1-3* mRNAs, allowing quantitation of the mRNA levels.

when provided on a low-copy centromere (CEN) plasmid in a *nrd1*Δ*39-575* strain derived from a different background. *nrd1-1* does not support viability when provided on a CEN plasmid, but viability is restored when it is expressed from a high-copy (2μ) plasmid. *nrd1-2* supports very weak viability when provided on a CEN plasmid, and slightly improved viability when provided on a 2μ plasmid.

A possible resolution was suggested by the observation that viability of the *nrd1-3* mutant is dependent on the downstream portion of the reading frame between codon 169 and the normal UAA termination signal at codon 576 (Fig. 5*A*, *nrd1-3* Δ*Bam-Bgl*). These results could be explained if translational readthrough of the premature stop codons in *nrd1-1*, *nrd1-2,* and *nrd1-3* is required for viability. The strain-dependent viability of *nrd1-1* and *nrd1-2* might result from differential efficiencies of readthrough in the two strains.

To explore this possibility, we monitored the production of full-length Nrd1 protein from these nonsense alleles by immunoblotting with polyclonal Nrd1 antiserum (Fig. 5*B*). The 64-kDa product corresponding to full-length Nrd1 protein in the *NRD1* parent strain, 46α, is greatly reduced in abundance in the *nrd1-1* and *nrd1-2* strains. However, some full-length protein is detectable in both of these strains, consistent with a low level of translational readthrough of their UAA (*nrd1-1*) and UAG (*nrd1-2*) stop codons. The expected 32-kDa product of *nrd1-2* is also detected, but the expected 27-kDa product of *nrd1-1*, which we infer from the dominant suppression results must be present, is not detected by antiserum raised against $Nrd1_{169–560}$.

Strikingly, virtually undiminished full-length Nrd1 protein accumulates in the *nrd1-3* strain (Fig. 5*B*, lane 4). Introduction of *nrd1-3* on a CEN plasmid into the *nrd1*Δ strain EJS101–9d also allows accumulation of abundant full-length protein (compare lanes 5 and 6), indicating that readthrough is not due to additional mutations in the original strain background. Primer extension analysis of the *nrd1-3* mRNA in the presence of ddATP confirmed the presence of a U residue in the first position of codon 164, converting the CAA glutamine codon to a UAA termination codon (Fig. 5*C*). Sequencing of the *nrd1-3* DNA confirmed the identity of the two A residues (not shown). Quantitation of the *nrd1-3* mRNA by using ddCTP in the primer extension assay revealed that it accumulates to approximately 90% the level of wild-type mRNA. Therefore, barring an increase in the stability of the resultant full-length protein, the UAA codon in the *nrd1-3* mRNA must be read through at an efficiency approaching 100% to account for the accumulation of full-length Nrd1. We conclude that the viability of *nrd1-3*, and probably *nrd1-1* and *nrd1-2*, is dependent on translational readthrough of their premature termination codons.

DISCUSSION

RNA Binding by Nrd1. We previously proposed that Nrd1 binds sequence-specifically to the U6R* cis element to direct a decrease in expression of a reporter pre-mRNA in yeast. Our results provide strong experimental support for this prediction. The carboxyl-terminal half of Nrd1 binds tightly (K_d = 10 nM) to a portion of the U6R* RNA containing a 14-nt subelement defined by point-mutant suppressors of down-regulation. These cis-suppressor mutations reduce Nrd1 binding to various degrees, with good correlation between the affinity of the RNA for Nrd1 *in vitro* and the efficiency of down-regulation *in vivo.* Our results suggest that as-yet-unidentified naturally occurring RNA sequences that bind to Nrd1 with K_d values in the 10–100 nM range may direct Nrd1-dependent regulation.

Other features of the U6R* element both 5′ and 3′ of the 14-nt cis-suppressor region also play important but undefined roles in down-regulation. These sequences may provide additional binding sites for Nrd1 or for other cellular factors such

as Sen1 (1), or structural features that render the 14-nt segment accessible for rapid recognition by Nrd1 or susceptible to the subsequent truncation of the pre-mRNA. Gel-shift experiments with RNAs containing the 5′ flanking sequence indicate that an additional high-affinity Nrd1-binding site is provided by this region. Indeed, the junction between the antisense U6 sequence and the 5′ flanking element creates a sequence, GUAAAACG, that is identical to the sequence encompassing the strongest cis-suppressor mutations (Fig. 1). However, no spontaneous cis-suppressor mutations were obtained in the junction sequence. Further mutational studies will be required to determine if the presence of a second high-affinity Nrd1-binding site at the junction of the 5′ flanking element and antisense U6 can account for the requirement for the 5′ flanking element. A search of the yeast genome for the sequence GTAAAACG identifies several hundred matches, but no instances of two such elements in close proximity, and no apparent bias in their positioning within or adjacent to known or suspected genes. A more empirical approach—e.g., utilizing the selective *in vitro* RNA-binding activity of Nrd1—may be necessary to identify the natural targets of Nrd1.

Cotranscriptional Function of Nrd1. A second critical feature of our model is the requirement for early recognition of the U6R* sequence element, before its removal from the pre-mRNA by splicing of the intron that harbors it. We have verified by indirect immunofluorescence that Nrd1 is localized to the nucleus, as is minimally required by this constraint (unpublished observations). In addition, we have provided strong evidence that an amino-terminal portion of Nrd1 implicated in binding to the CTD of RNA Pol II is important for down-regulation. Such an interaction may facilitate cis-element binding by localizing Nrd1 in proximity to the emerging transcript. Interaction with the RNA Pol II CTD could also play an important role in directing the subsequent generation of RNA 3′ ends, which may be a product of transcription termination by the polymerase.

Considerable interest has focused recently on the discovery that the RNA Pol II CTD interacts with the processing machineries responsible for capping, and at least in mammalian cells, splicing and 3′-end maturation of pre-mRNA (reviewed in refs. 3 and 6–8). Interestingly, a yeast ORF that we showed previously has amino-terminal sequence similarity to the Nrd1 CTD-binding motif (1) has recently been found to code for Pcf11, a subunit of cleavage and polyadenylation factor I (9), suggesting that 3′-end maturation in yeast may be transcriptionally coupled as well. While our results define a functional role for the CTD-binding motif of Nrd1 in down-regulation of the U6R*-containing pre-mRNA, this motif is dispensable for viability. Thus the essential cellular function of Nrd1, presumably dependent on RNA-binding activity, may be manifest at a later stage in the life cycle of its target RNAs.

Our original conclusion that suppression of pre-mRNA down-regulation by the *nrd1-1*, *nrd1-2,* and *nrd1-3* mutations is due to the absence of an intact RNA-binding domain appears to have been an oversimplification. The premature stop codons present in the *nrd1-1* and *nrd1-2* alleles do result in greatly reduced expression of full-length protein, probably accounting at least in part for their suppressor phenotypes. However, the dominant suppression of down-regulation afforded by overexpression of the truncated products of these alleles indicates that a similar effect may be operating in the original suppressor strains, particularly in light of the greatly reduced abundance of full-length Nrd1 protein in these strains. The most likely mechanism for dominant suppression is competition with full-length Nrd1 protein for binding to the RNA Pol II CTD by the amino-terminal fragment of Nrd1, which cannot recognize the cis element in the nascent pre-mRNA.

Readthrough of the *nrd1-3* Stop Codon. Understanding the suppression of RNA down-regulation by the *nrd1-3* mutation requires clarification of the mechanism whereby abundant full-length Nrd1 protein is produced despite the presence of an in-frame UAA at codon 164. Three mechanisms for "recoding" of stop codons with maintenance of reading frame have been defined (10): readthrough with insertion of a standard amino acid, directed by near-cognate tRNA; "ribosome hopping", with stop codon bypass due to large-scale slippage of the mRNA with respect to the peptidyl-tRNA specified by the preceding codon; and readthrough with insertion of selenocysteine, a special case occurring only for certain UGA codons. The first of these is most plausible for *nrd1-3*, although we cannot rule out a short "hop" from the UCA codon for serine preceding the stop codon to a UCA four codons downstream. Whatever the mechanism, the resulting substitution or deletion of amino acid(s) may be responsible for the suppression of pre-mRNA down-regulation and for the temperature-sensitive growth defect of *nrd1-3.*

Readthrough of UAA at codon 164 in *nrd1-3* is context-dependent, since UAA at codon 242 in *nrd1-1* yields much less full-length protein, and UAA at the normal termination site promotes efficient termination in *nrd1-3*. In a systematic study of context effects on termination efficiency in *Saccharomyces cerevisiae* (11), UAA was read through at a frequency of 13% when flanked on both sides by CAA codons for glutamine, but only 1% when just the downstream CAA codon was present, as in *nrd1-3*. Context effects beyond the immediate flanking codons, possibly involving local mRNA structure (10), may be at play in the efficient recoding of UAA in *nrd1-3*. Additional studies will be needed to determine the mechanism responsible for readthrough of UAA at codon 164 and the role of amino acid identity at this position in Nrd1-mediated down-regulation.

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1. Steinmetz, E. J. & Brow, D. A. (1996) *Mol. Cell. Biol.* **16,** 6993–7003.
2. Yuryev, A., Patturajan, M., Litingtung, Y., Joshi, R. V., Gentile, C., Gebara, M. & Corden, J. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6975–6980.
3. Steinmetz, E. J. (1997) *Cell* **89,** 491–494.
4. Gill, S. C. & von Hippel, P. H. (1989) *Anal. Biochem.* **182,** 319–326.
5. Wise, J. A. (1991) *Methods Enzymol.* **194,** 405–415.
6. Corden, J. L. & Patturajan, M. (1997) *Trends Biochem. Sci.* **22,** 413–416.
7. Neugebauer, K. M. & Roth, M. B. (1997) *Genes Dev.* **11,** 3279–3285.
8. Shuman, S. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12758–12760.
9. Amrani, N., Minet, M., Wyers, F., Dufour, M. E., Aggerbeck, L. P. & Lacroute, F. (1997) *Mol. Cell. Biol.* **17,** 1102–1109.
10. Gesteland, R. F. & Atkins, J. F. (1996) *Annu. Rev. Biochem.* **65,** 741–768.
11. Bonetti, B., Fu, L., Moon, J. & Bedwell, D. M. (1995) *J. Mol. Biol.* **251,** 334–345.